

Supplementary Information for

Hyaluronic acid-bilirubin nanomedicine for targeted modulation of dysregulated intestinal barrier, microbiome, and immune responses in colitis.

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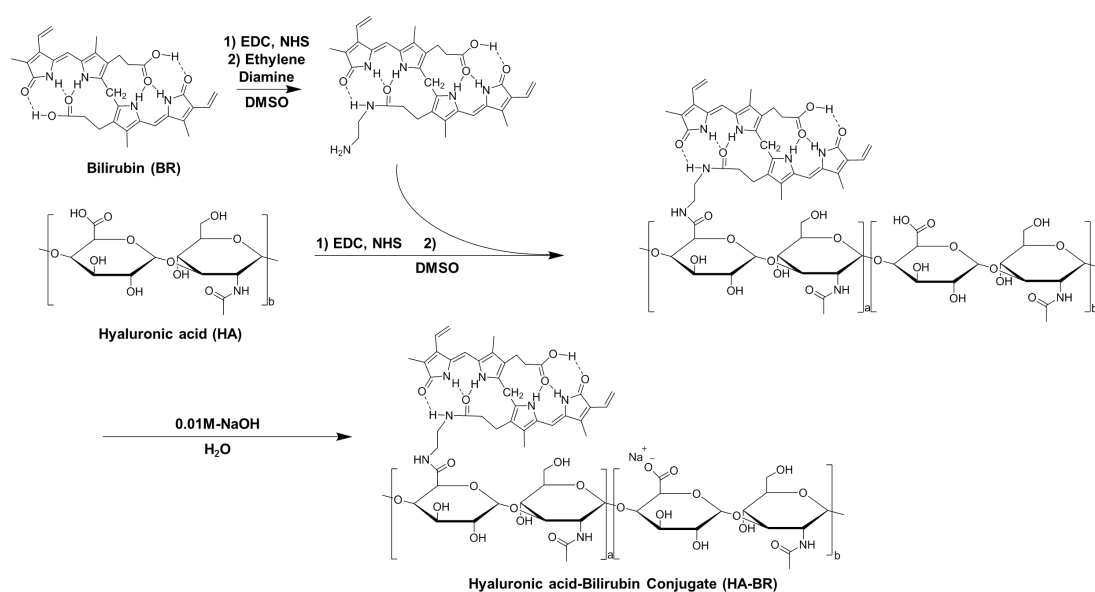
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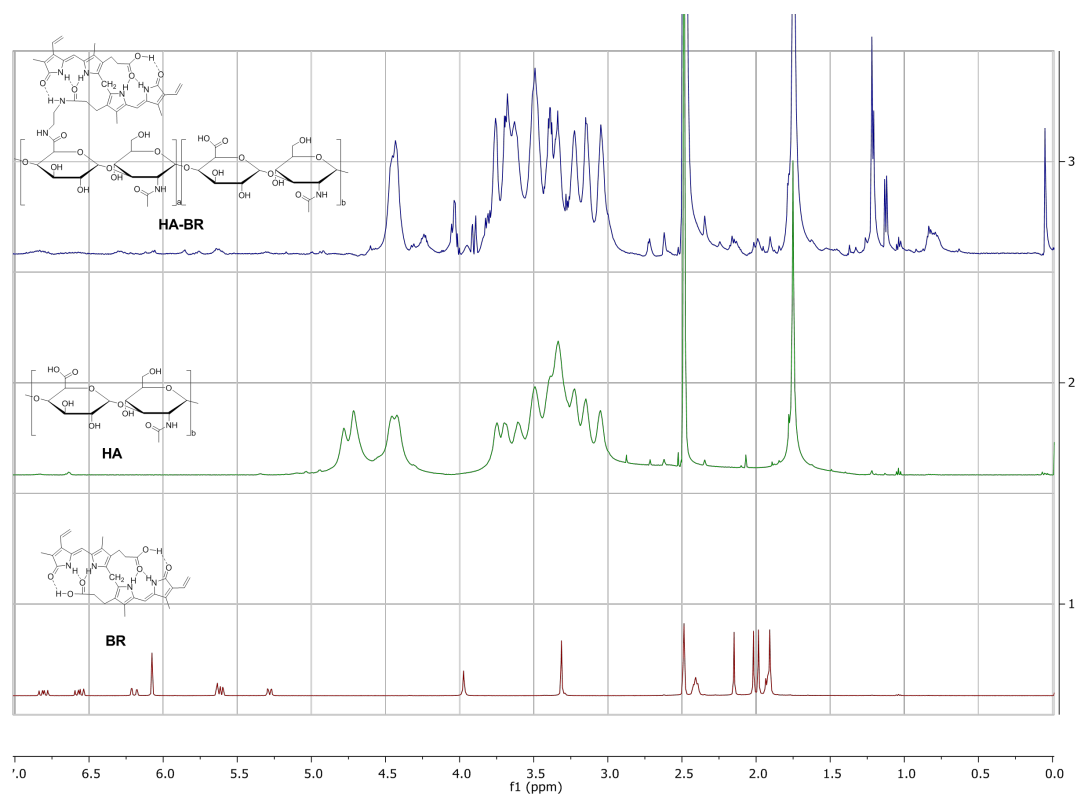
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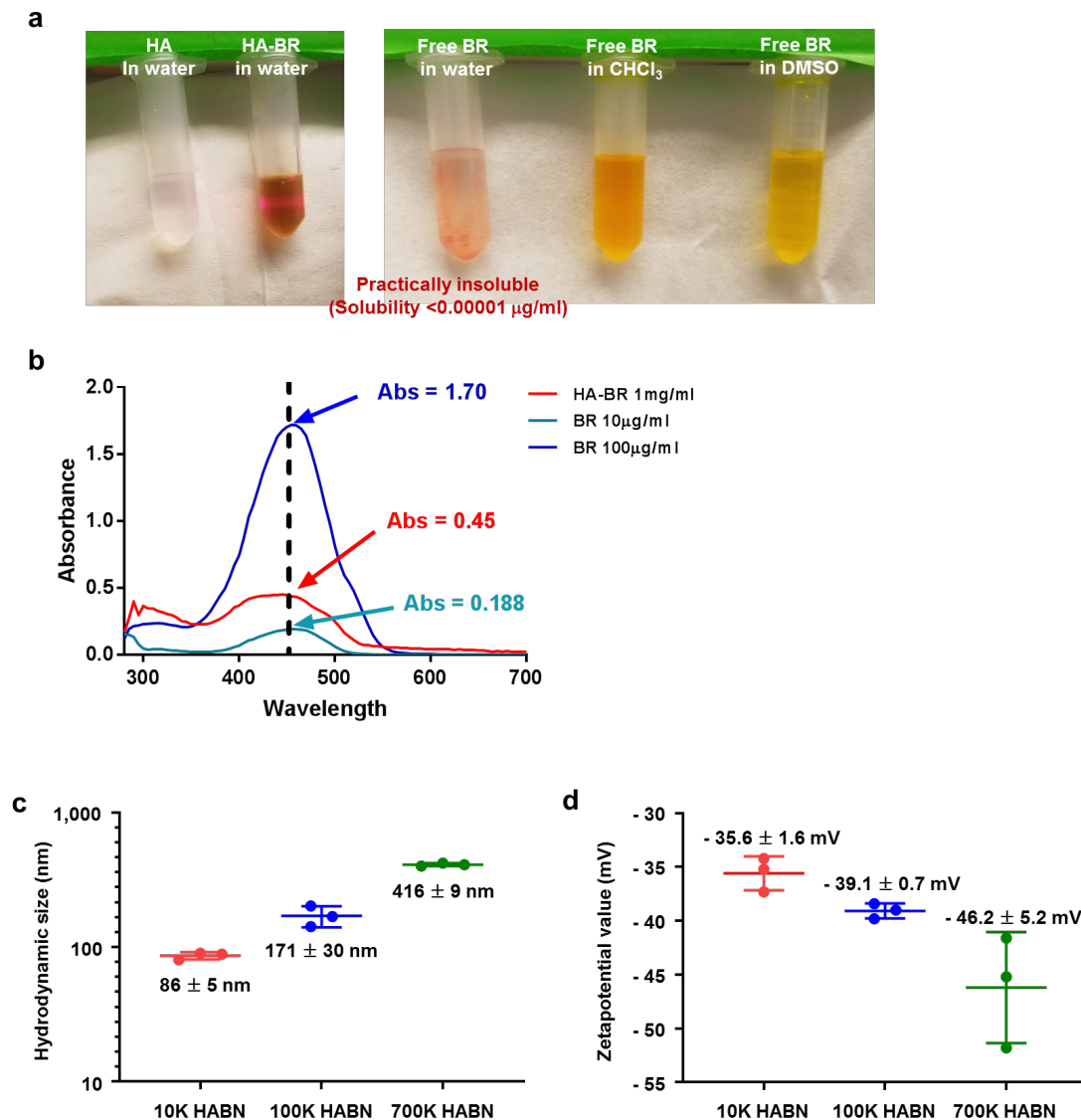
Supplementary Figures



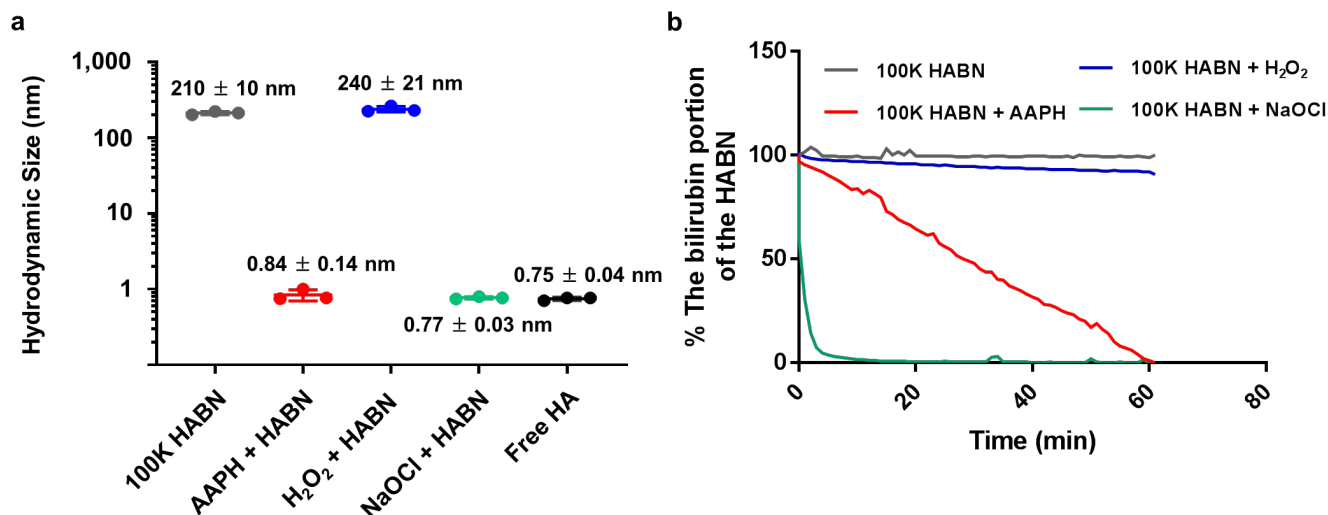
Supplementary Figure 1. A scheme for the synthesis of hyaluronic acid-bilirubin conjugate (HA-BR).



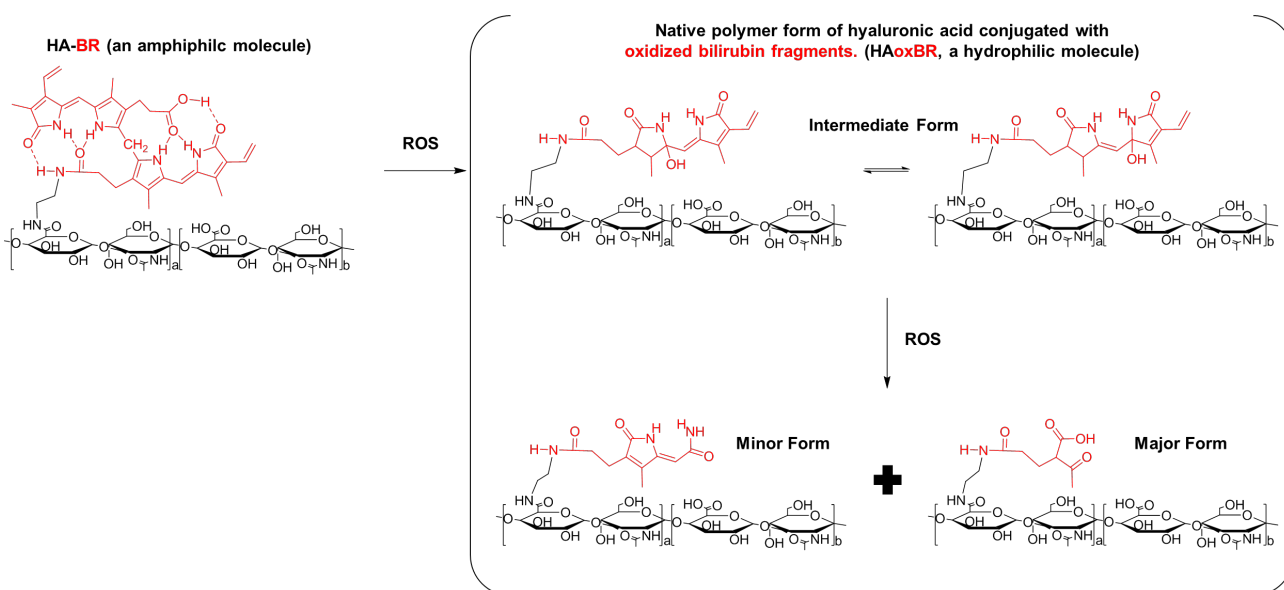
Supplementary Figure 2. NMR spectra of HA-BR, hyaluronic acid (HA), and bilirubin (BR).



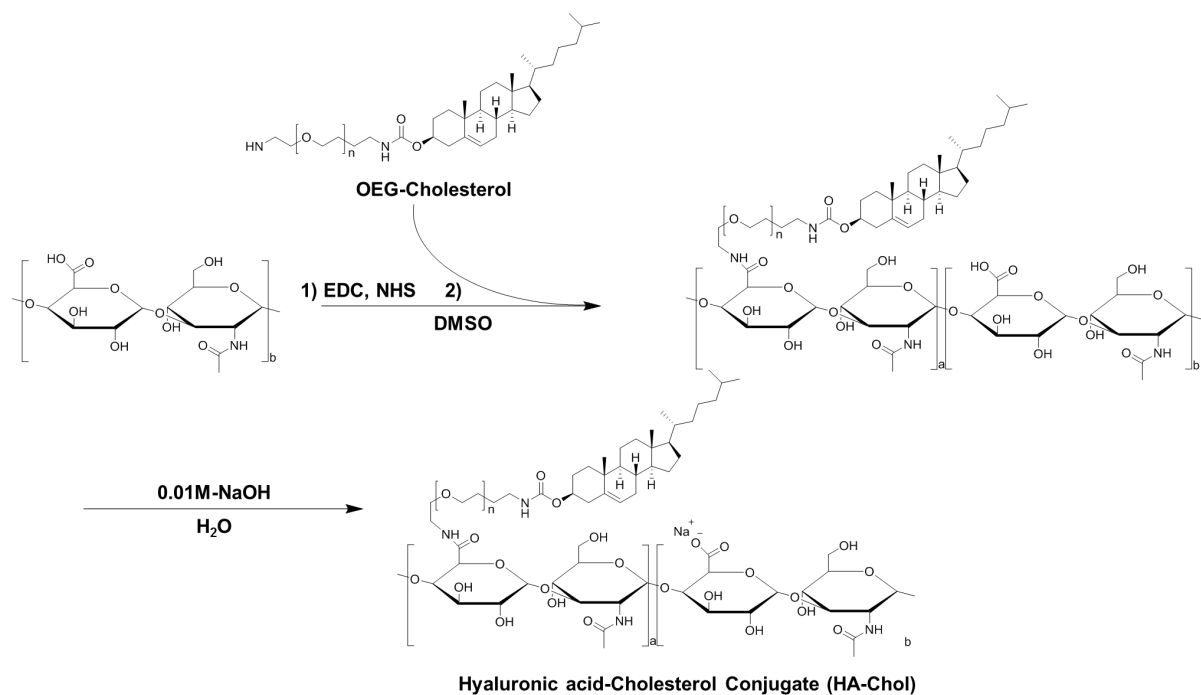
Supplementary Figure 3. Characterization of HA-BR and HABN. **a**, Solubility or dispersibility of HA (3 mg/ml), BR (100 μg/ml), or HABN (3 mg/ml), in water, CHCl₃, or DMSO. **b**, UV/Vis spectra of HABN (1 mg/ml; BR 25 μg/ml in suspension) and BR (10 μg/ml and 100 μg/ml). **c-d**, Hydrodynamic sizes (**c**) and zeta potential values (**d**) of 10K HABN, 100K HABN, and 700K HABN. Data are presented as mean ± s.d. from a representative experiment (n = 3 biologically independent samples) from 2 independent experiments.



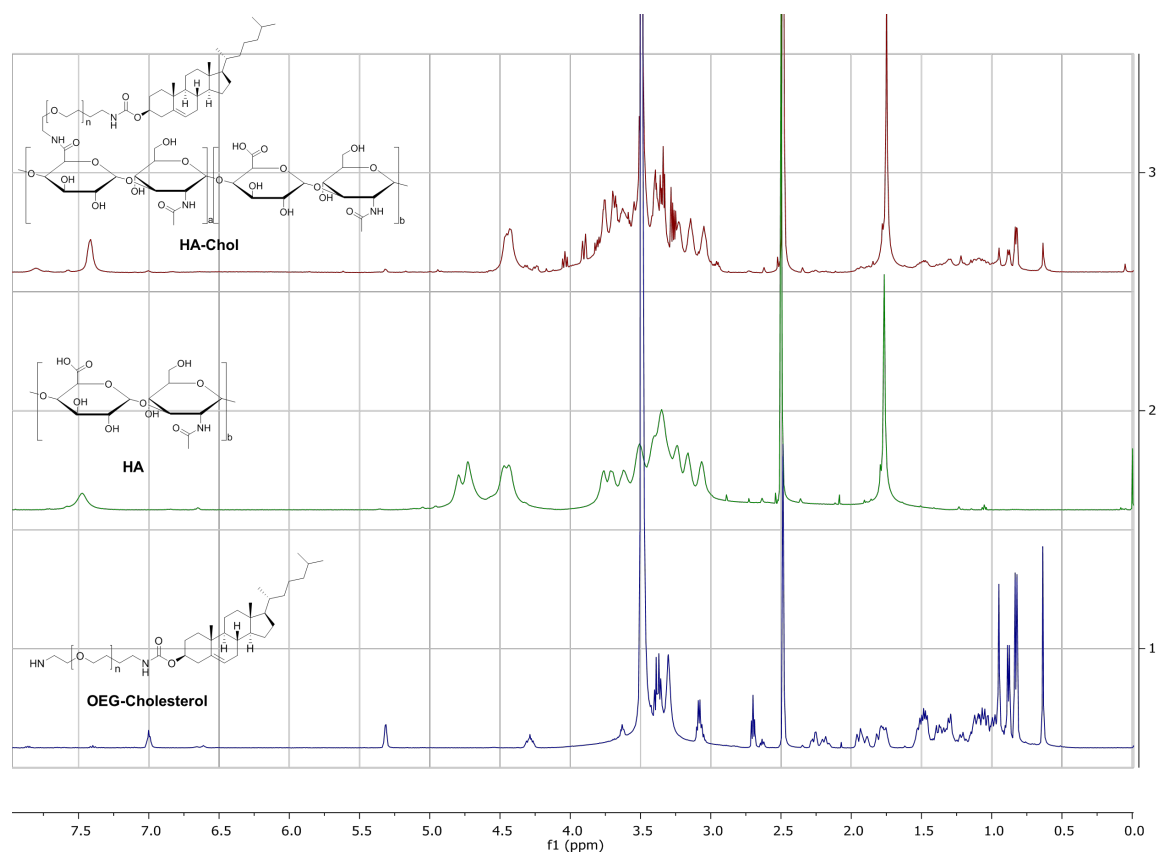
Supplementary Figure 4. ROS-mediated decomposition of HABN. **a**, Changes in the size of HABNs (in PBS) induced by ROS stimuli, as determined by dynamic light scattering. HABNs were exposed to a peroxy radical generator AAPH (100 mM), H₂O₂ (5 mM), or NaOCl (1 mM). **b**, UV/VIS spectra of 100K HABN (1 mg/ml) in PBS treated with 100 mM of AAPH, 5 mM of H₂O₂, or 1 mM of NaOCl over 1 h. Data are presented as mean ± s.d. from a representative experiment (n = 3 biologically independent samples) from 2 independent experiments.



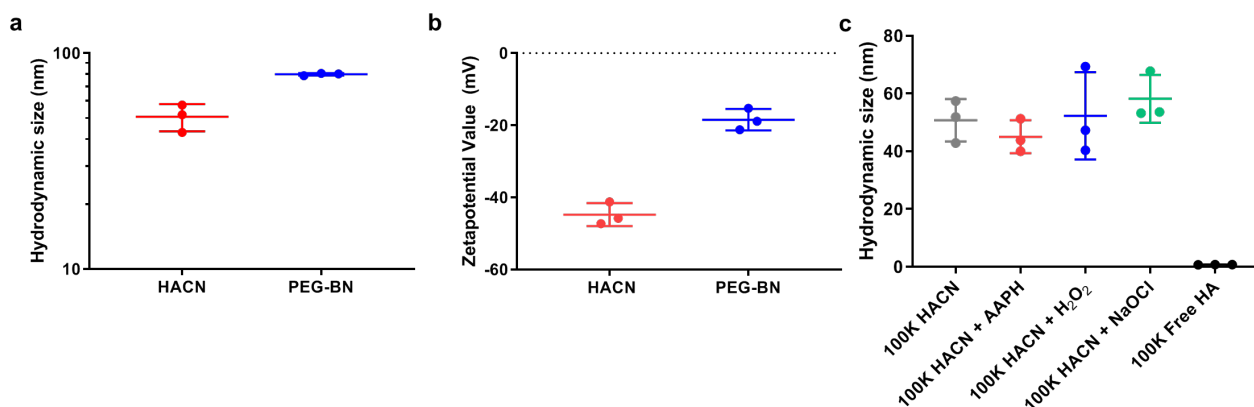
Supplementary Figure 5. A scheme for oxidative degradation of HA-BR.



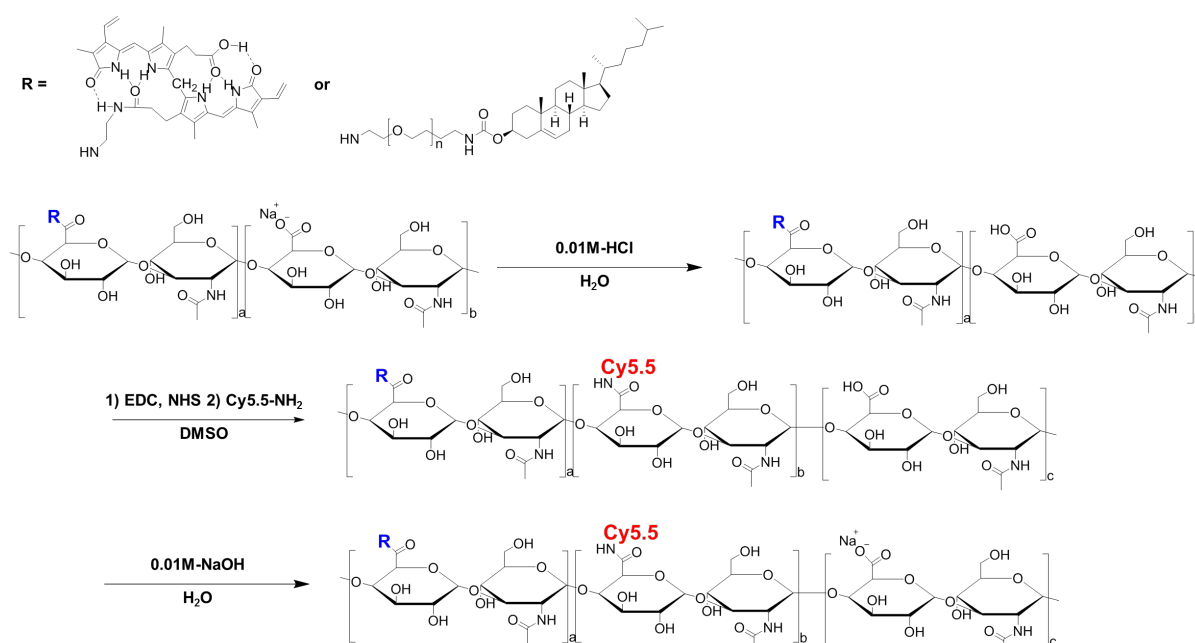
Supplementary Figure 6. A scheme for the synthesis of hyaluronic acid-cholesterol conjugate (HA-Chol).



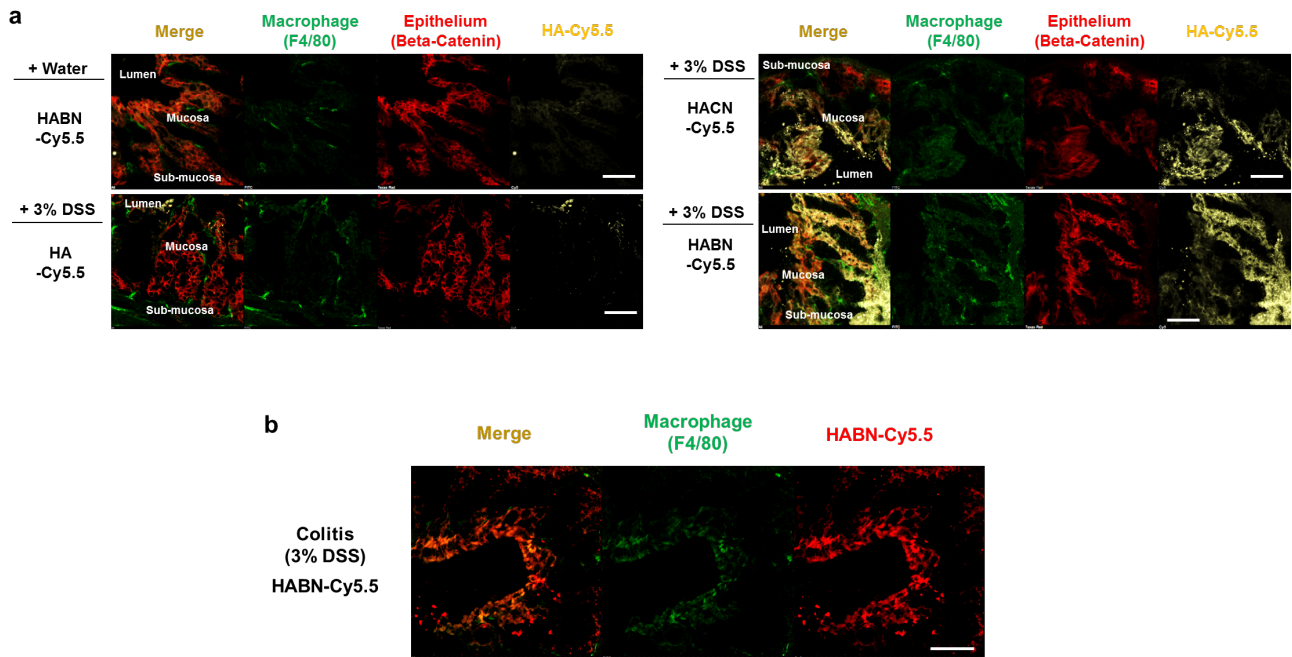
Supplementary Figure 7. NMR spectra of HA-Chol, HA, and OEG-Cholesterol.



Supplementary Figure 8. Characterization of HACN and PEG-BN. **a-b**, hydrodynamic sizes (**a**) and zeta potential values (**b**) of hyaluronic acid-cholesterol nanoparticle (HACN) and PEGylated bilirubin nanoparticle (PEG-BN). Data are presented as mean \pm s.e.m. from a representative experiment ($n = 3$) from 2 independent experiments. **c**, HACNs exposed to AAPH (100 mM), H₂O₂ (5 mM), or NaOCl (1 mM) for 1 h were analyzed by dynamic light scattering. Data are presented as mean \pm s.d. from a representative experiment ($n = 3$ biologically independent samples) from 2 independent experiments.

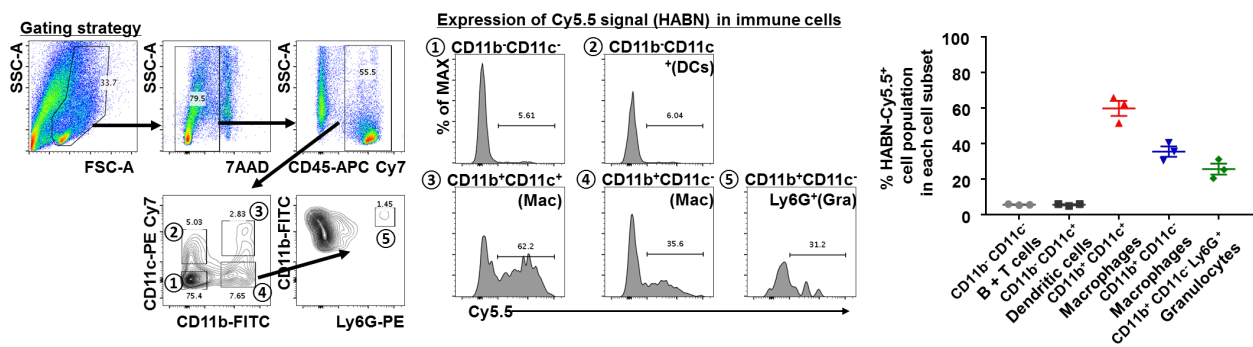


Supplementary Figure 9. A scheme for the synthesis of HABN-Cy5.5 and HACN-Cy5.5.

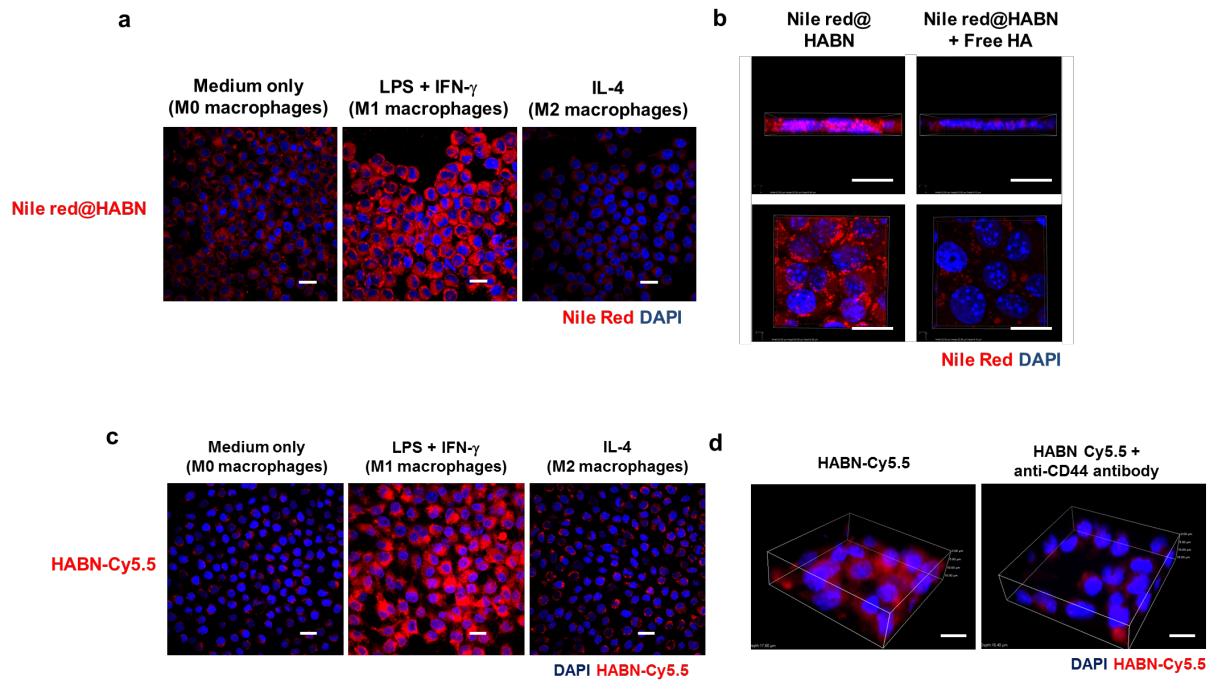


Supplementary Figure 10. Accumulation of HABN in macrophages within DSS-treated inflamed colon.

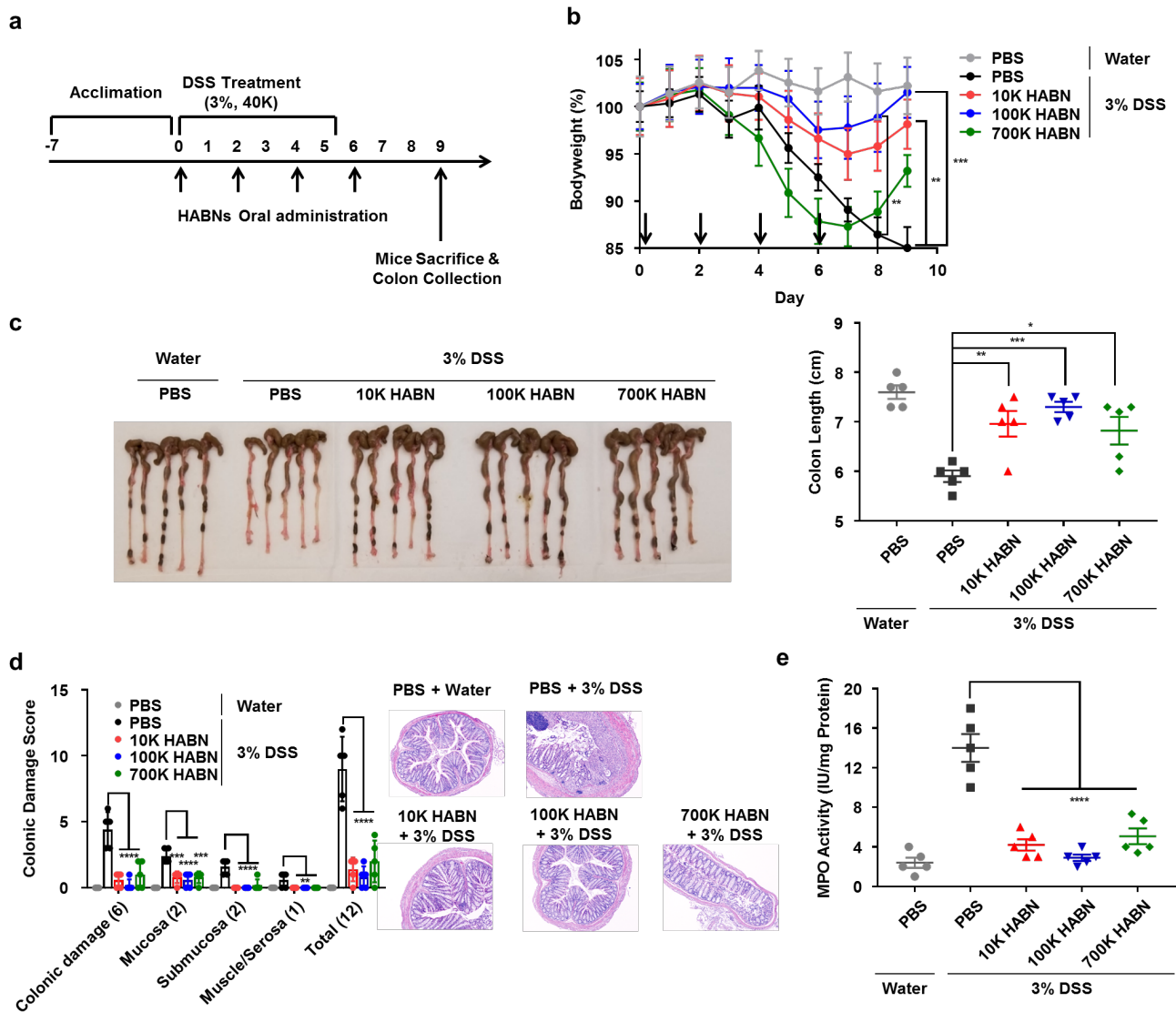
a, Healthy or DSS-colitis mice were orally administered on day 6 with 7.5 mg/kg of HA-Cy5.5, HCN-Cy5.5, or HABN-Cy5.5 (equivalent mass of Cy5.5), and colon tissues were excised after 6 h, stained with anti-F4/80 and anti- β -catenin antibodies, and visualized by confocal microscopy. Scale bars = 50 μ m. **b**, Colon tissues obtained from mice given 3% DSS water for 6 days and orally administered with HABN-Cy5.5 (7.5 mg/kg) on day 6 were stained with anti-F4/80 antibody and visualized by confocal microscopy. Scale bar = 50 μ m. Shown are representative images from 6 slides with $n = 3$ biologically independent animals from 2 independent experiments.



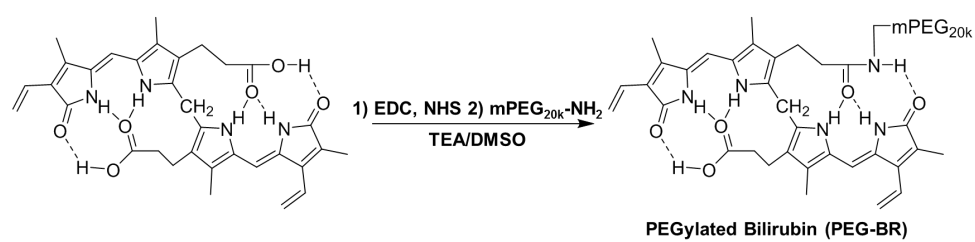
Supplementary Figure 11. Accumulation of HABN in macrophages in the lamina propria. After lamina propria mononuclear cells were incubated *in vitro* with 10 μ g/ml HABN-Cy5.5 for 3 h, particle uptake was analyzed by flow cytometry. Data are presented as mean \pm s.e.m. from a representative experiment ($n = 3$ biologically independent samples) from 2 independent experiments.



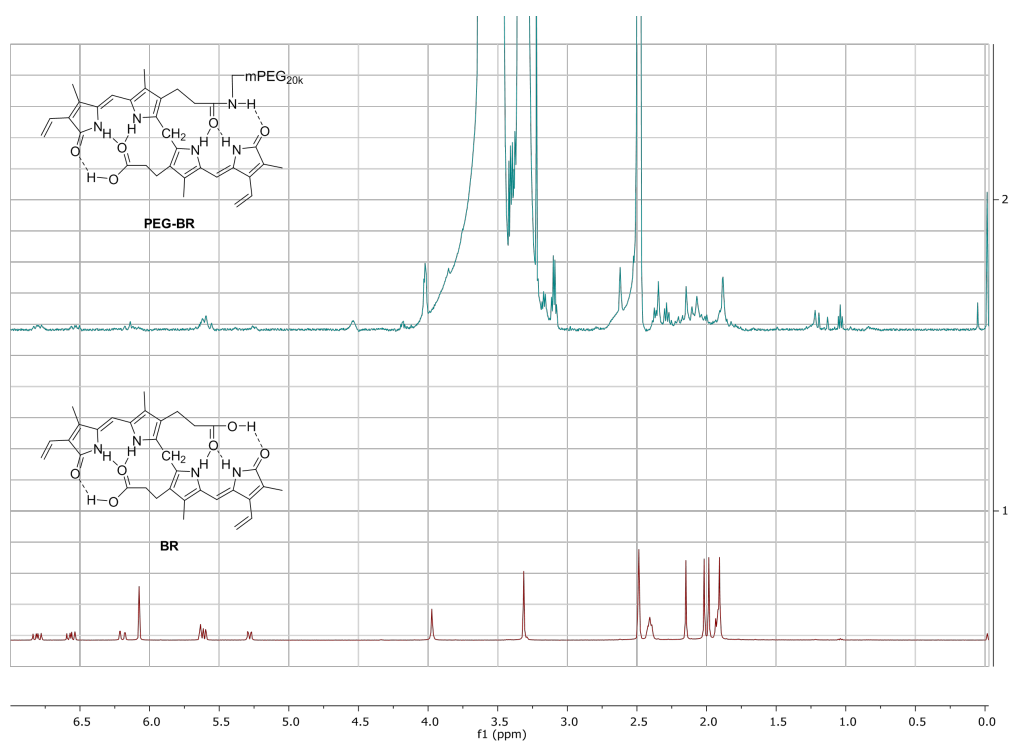
Supplementary Figure 12. CD44-HA interactions mediate association of HABN-Cy5.5 among M1 macrophages. **a**, Confocal microscopy XYZ images of J774A.1 macrophage cells pre-treated for 24 h with culture medium, LPS (100 ng/ml) and IFN- γ (10 ng/ml), or IL-4 (20 ng/ml), followed by 1 hr incubation with Nile red@HABN (Nile Red, 100 ng/ml; HABN 2.5 μ g/ml). **b**, M1-type macrophages were pre-treated for 1 h with or without free HA (5 mg/ml). DAPI was used for nuclei counter staining. Scale bars = 20 μ m (**a**) or 15 μ m (**b**). Confocal microscopy XYZ images of J774A.1 macrophage cells pre-treated for 24 h with culture medium, LPS (100 ng/ml) and IFN- γ (10 ng/ml), or IL-4 (20 ng/ml), followed by 1 hr incubation with 100K HABN-Cy5.5 (5 μ g/ml). M1-type macrophages were pre-treated for 1 h with anti-CD44 antibody (500 μ g/ml). **c**, J774A.1 macrophage cells were pre-treated with culture medium, LPS (100 ng/ml) and IFN- γ (10 ng/ml), or IL-4 (20 ng/ml) for 24 h and incubated with HABN-Cy5.5 (5 μ g/ml) for 1 h, followed by confocal microscopy. **d**, J774A.1 cells were pre-treated with LPS (100 ng/ml) and IFN- γ (10 ng/ml) for 24 h, incubated with or without anti-CD44 antibody (500 μ g/ml) for 1 h, followed by treatment with HABN-Cy5.5 (5 μ g/ml) for 1 h. Scale bars = 15 μ m (**a-b**), 20 μ m (**c**), or 15 μ m (**d**). DAPI was used for nuclei counter staining. Scale bar = 15 μ m. Shown are representative images from 6 slides with $n = 3$ biologically independent samples from 2 independent experiments.



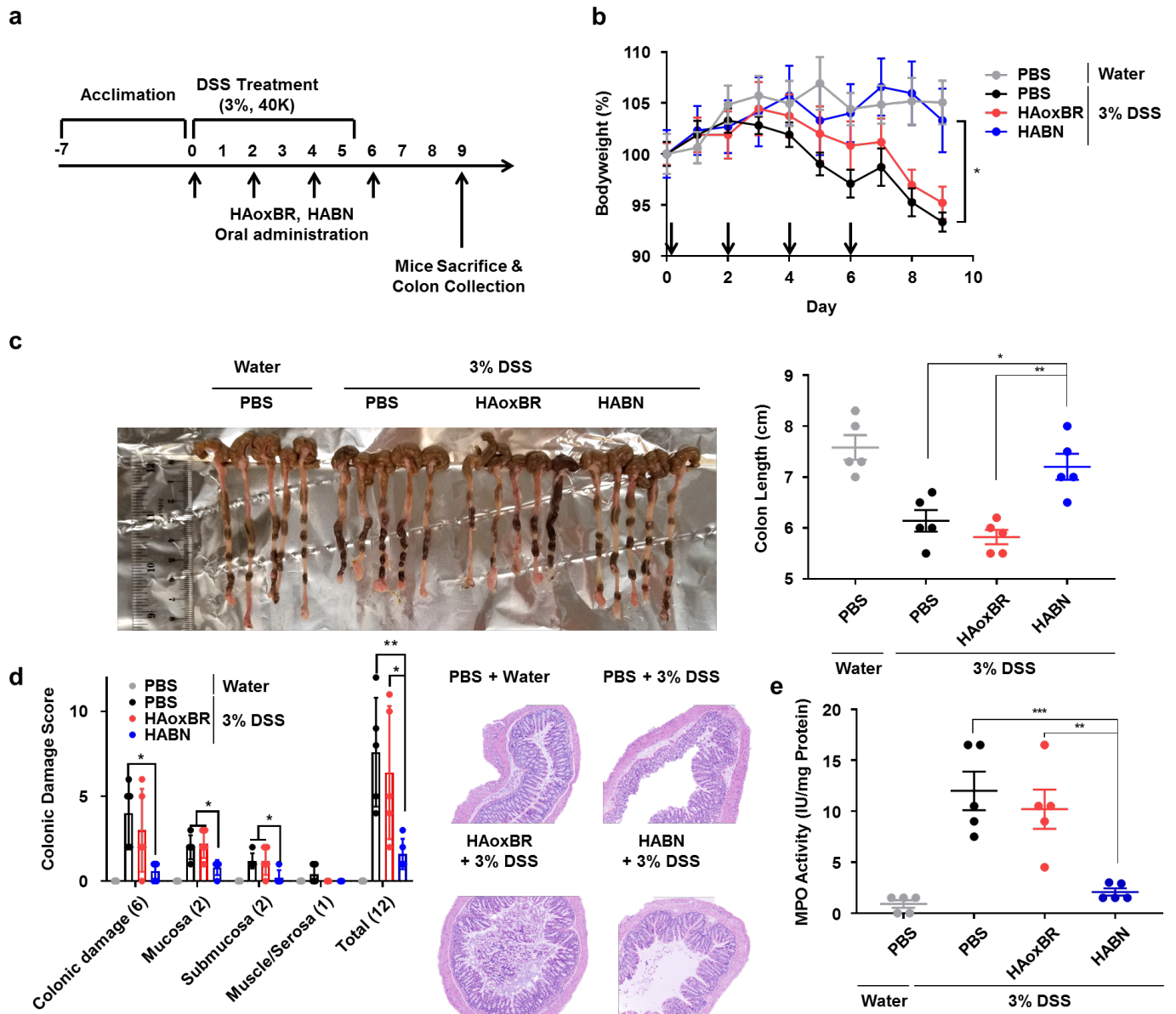
Supplementary Figure 13. HABN ameliorates colonic damage in a murine model of colitis. **a**, C57BL/6 mice were provided with 3% DSS in drinking water for 6 days. On days 0, 2, 4, and 6, the animals were orally administered with PBS or 30 mg/kg of HABNs (10K, 100K, or 700K, equivalent mass of HA). **b**, Daily bodyweight changes in each group for 9 days. **c**, Colon length was measured on day 9, and **d**, colon sections stained with hematoxylin and eosin (H&E) were analyzed for colonic damage scores. **e**, Colonic MPO activity was measured. Data are presented as mean \pm s.e.m. from a representative experiment ($n = 5$ biologically independent animals) from 2 independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, analyzed by (**c,d,e**) one-way or (**b**) two-way ANOVA with Tukey's HSD multiple comparison post hoc test.



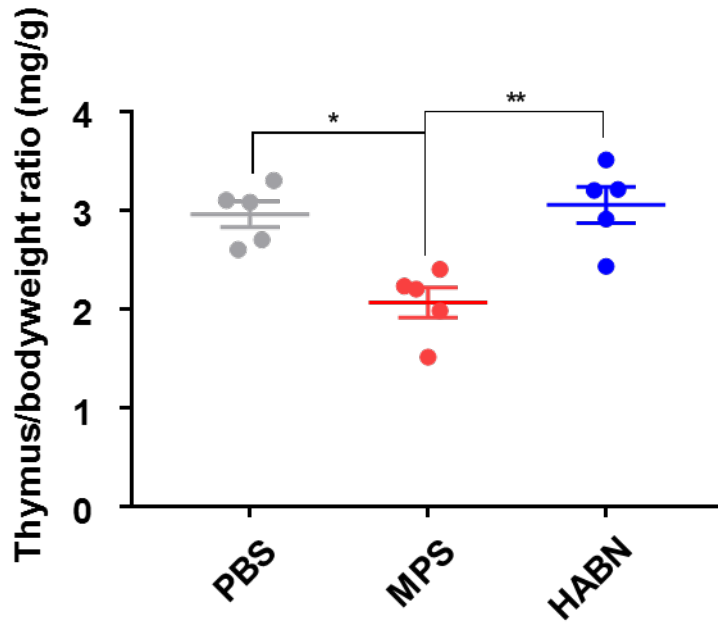
Supplementary Figure 14. A scheme for the synthesis of PEGylated bilirubin (PEG-BR).



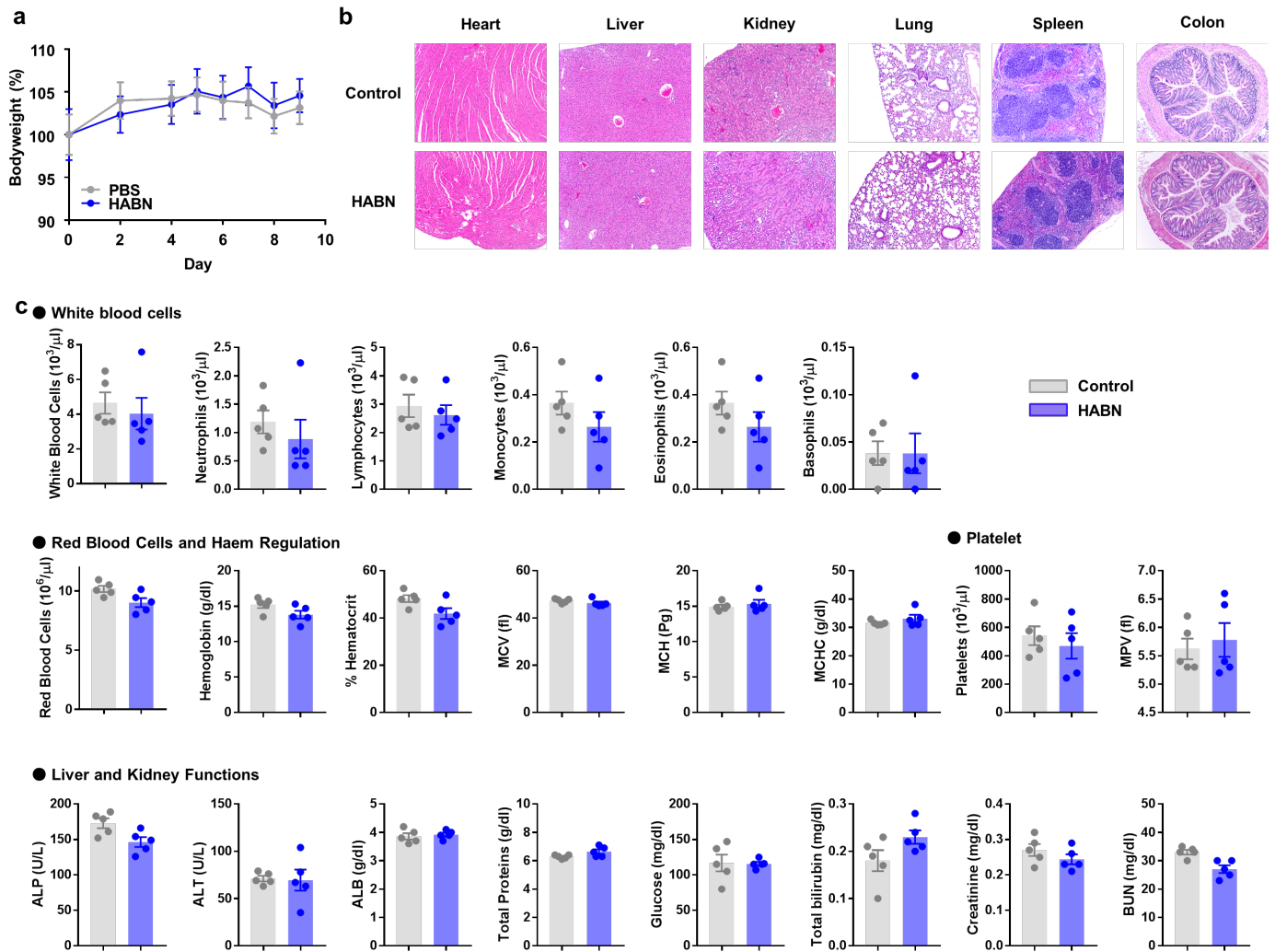
Supplementary Figure 15. NMR spectra of PEG-BR and BR.



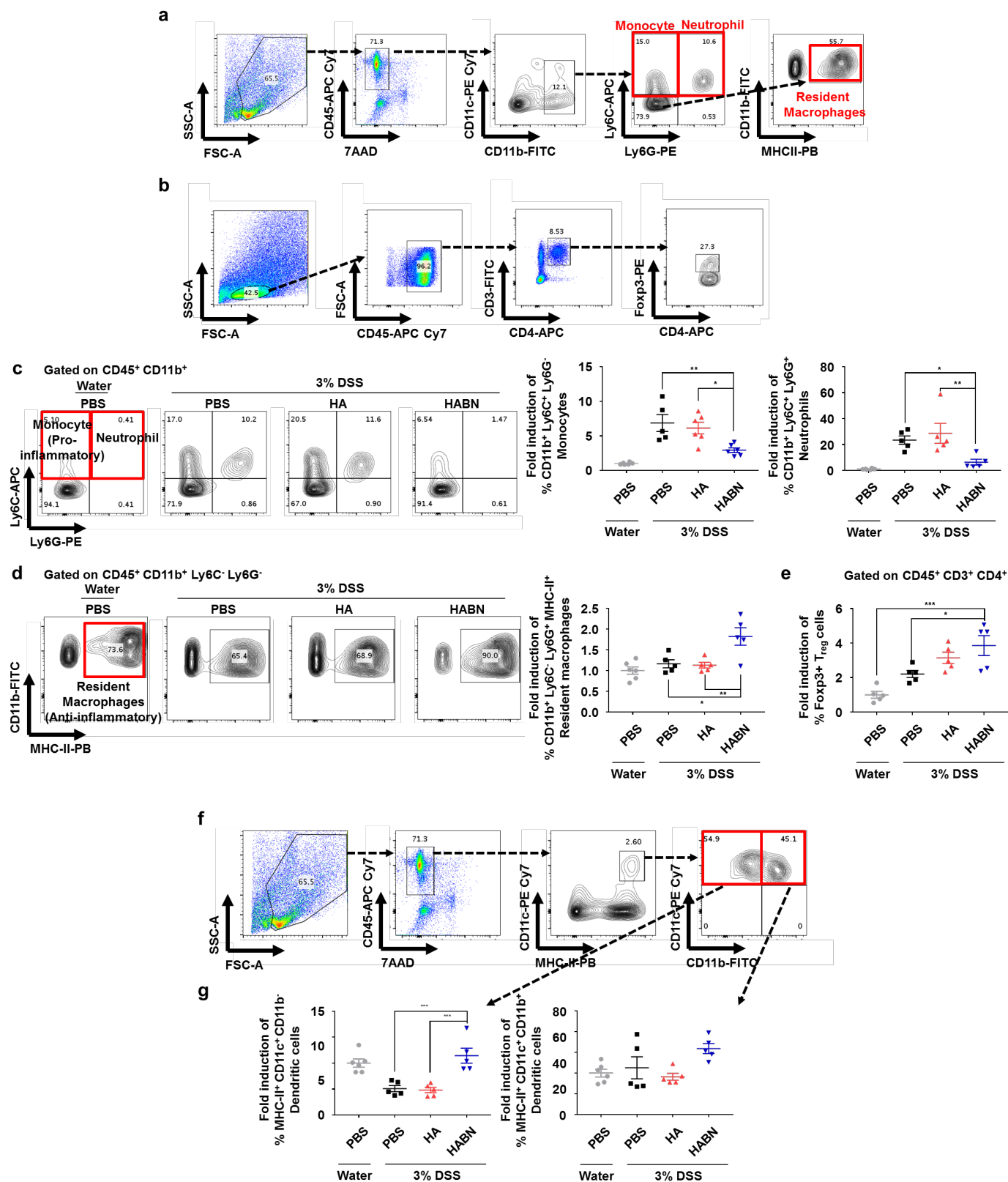
Supplementary Figure 16. HABN, but not HAoxBR, protects animals against DSS-induced colitis. **a**, C57BL/6 mice were provided with water or 3% DSS-containing water for 6 days. On days 0, 2, 4, and 6, mice were orally administered with PBS or 30 mg/kg of HABN or HAoxBR. **b**, Daily bodyweight changes in each group for 9 days. **c-e**, On day 9, animals were euthanized, and (**c**) colon length, (**d**) colonic damage scores, and (**e**) colonic MPO activity were measured. Data are presented as mean \pm s.e.m. from a representative experiment ($n = 5$ biologically independent animals) from 2 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed by (**c,e**) one-way or (**b,d**) two-way ANOVA with Tukey's HSD multiple comparison post hoc test.



Supplementary Figure 17. Whereas MPS induces thymus involution, HABN does not affect thymus weight. a, C57BL/6 mice were orally administered with PBS or 1 mg/kg of MPS or 30 mg/kg of HABN every day for 6 days and on day 7, a ratio of thymus to bodyweight was measured. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed by one-way ANOVA with Tukey's HSD multiple comparison post hoc test. Data are presented as mean \pm s.e.m. from a representative experiment ($n = 5$ biologically independent animals) from 2 independent experiments.

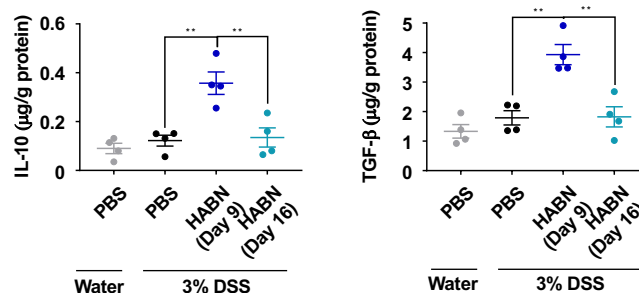


Supplementary Figure 18. Safety profiles of HABN. **a-c**, Mice were orally administered on day 0, 2, 4, and 6 with 30 mg/kg of HABN or PBS, and on day 9, blood and major organs were collected for blood hematology, blood chemistry, and histology analysis. **a**, Daily bodyweight changes in each group for 9 days **b**, Major organ (heart, liver, kidney, lung, spleen, and colon) sections stained hematoxylin and eosin (H&E) were analyzed for systemic toxicity evaluation. **c**, Blood were analyzed using blood hematology and blood chemistry for systemic toxicity evaluation. Data are presented as mean \pm s.e.m. from a representative experiment ($n = 5$ biologically independent animals) from 2 independent experiments.



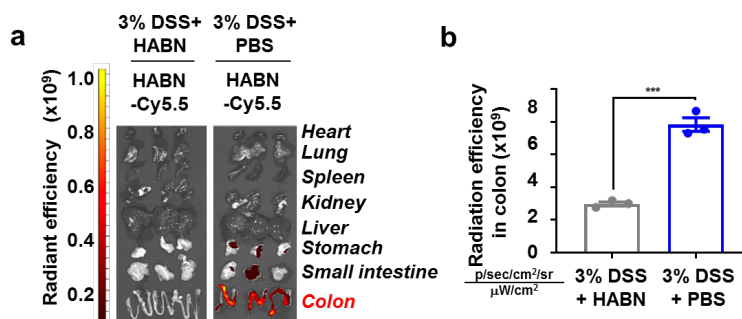
Supplementary Figure 19. Analysis of lamina propria mononuclear cells in colon. **a-g**, DSS-colitis mice were orally administered on day 0, 2, 4, and 6 with PBS or 30 mg/kg of HA, HACN, or HABN (equivalent mass of HA), and on day 9, their lamina propria in colon were analyzed for the frequencies of immune cells populations. **a**, Gating strategy for analyzing pro-inflammatory CD45⁺CD11b⁺Ly6C⁺Ly6G⁻ monocytes, CD45⁺CD11b⁺Ly6C⁺Cy6G⁺ neutrophils, and anti-inflammatory CD45⁺CD11b⁺Ly6C⁻Ly6G⁻MHCII⁺ resident macrophages. **b**, Gating strategy for analyzing CD45⁺CD4⁺CD8⁻FOXP3⁺ Treg cells. **c-e**, Frequencies of (**c**) pro-inflammatory CD45⁺CD11b⁺Ly6C⁺Ly6G⁻ monocytes and CD45⁺CD11b⁺Ly6C⁺Cy6G⁺ neutrophils; (**d**) anti-

inflammatory CD45⁺CD11b⁺Ly6C⁺Ly6G⁺MHCII⁺ resident macrophages; and (e) CD45⁺CD4⁺CD8⁺Foxp3⁺ Treg cells. f, Gating strategy for analyzing CD45⁺CD11c⁺MHCII⁺CD11b⁺ dendritic cells and CD45⁺CD11c⁺MHCII⁺CD11b⁺ dendritic cells and g, quantification of their frequencies. Data are presented as mean \pm s.e.m. from a representative experiment (n = 5 biologically independent animals) from 2 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, analyzed by one-way ANOVA with Tukey's HSD multiple comparison post hoc test.



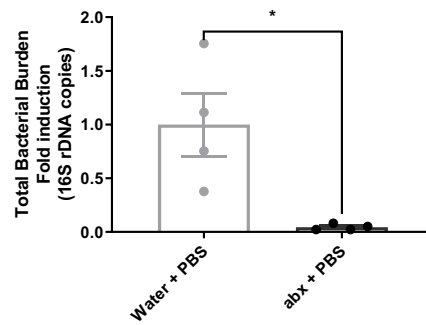
Supplementary Figure 20. HABN transiently elevates anti-inflammatory cytokines in the colon.

Healthy or DSS-colitis mice were orally administered on day 0, 2, 4, and 6 with PBS or 30 mg/kg of HABN. On day 9 or day 16, colon tissues were analyzed for the concentrations of anti-inflammatory cytokines (IL-10, TGF- β). ** p < 0.01, analyzed by one-way ANOVA with Tukey's HSD multiple comparison post hoc test. Data are presented as mean \pm s.e.m. from a representative experiment (n = 4 biologically independent animals) from 2 independent experiments.



Supplementary Figure 21. Accumulation of HABN in colon is significantly reduced after DSS-colitis mice were treated with HABN.

a-b, C57BL/6 mice were provided with 3% DSS-containing water for 6 days. On days 0, 2, 4, and 6, mice were orally administered with 30 mg/kg of HABN (with 100K HA at equivalent mass) or PBS. On day 9, animals were orally administered with 7.5 mg/kg of HABN-Cy5.5. After 6 h, their organs were imaged by IVIS (a) and quantified for Cy5.5 fluorescence signal (b). Data are presented as mean \pm s.e.m. from a representative experiment (n = 3 biologically independent animals) from 2 independent experiments. *** p < 0.001, analyzed by Student's unpaired, two-sided t-test.



Supplementary Figure 22. Broad-spectrum oral antibiotics disrupt gut microbiome. C57BL/6 mice were pre-treated for 5 days with a cocktail of antibiotics (ampicillin, metronidazole, vancomycin, and neomycin) added to the drinking water and then feces were analyzed for the total bacterial burden by measuring universal eubacterial DNA with qPCR. * $p < 0.05$, analyzed by Student's unpaired, two-sided t-test. Data are presented as mean \pm s.e.m. from a representative experiment ($n = 4$ biologically independent animals) from 2 independent experiments.